Culturing of Human Hepatocellular Carcinoma

A Simple and Reproducible Method

KINGKARN LAOHATHAI, MD, PhD,
and NATTH BHAMARAPRAVATI, MD, DSc

From the Liver Tissue Culture Unit, Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand

Eight permanent human hepatocellular carcinoma (HHC) cell lines were established from 8 individual patients by the use of aspirated needle biopsy specimens (smaller than 0.1 ml in size). The cells grew in clustered form and retained intercellular junctions and canaliculi resembling bile canaliculi. The presence of secreted human α-fetoprotein and human albumin was detected in the cultured medium. Hepatitis B surface (HBs) antigen was not found on these cells. Implantation of the cells into athymic mice was followed by the growth of hepatocellular carcinomas and the appearance of human α-fetoprotein in the mouse serum. Chromosome analysis of three of the cell lines showed hyperdiploidy in two of them and hypotetraploidy in the other. Enzyme analyses of culture medium and cell homogenates have detected some enzymes characteristic of liver tissue such as γ-glutamyl transferase, sorbital dehydrogenase, alkaline phosphatase, glutamate dehydrogenase, as well as aspartate and alanine transaminase. These tumor cells have been continuously maintained in culture for over 6 years with no significant changes observed. (Am J Pathol 1985, 118:203-208)

CELL LINES derived from human hepatocellular carcinoma (HHC) have been successfully used in many studies, such as for the determination of the presence of viral DNA,[1,2] hepatitis B surface (HBs) antigen,[3,4] and other immunologic aspects of HHC.[5] The probability exists that a variety of etiologic agents contribute to the development of HHC,[6-8] and these changes might be reflected and maintained in the cell culture lines, eg, the presence of oncogenic viruses or activated oncogenes.[9,10] In addition, recent studies by a number of groups have demonstrated the scientific and clinical significance of tumor heterogenity.[11,12] It was, therefore, advantageous to acquire an efficient means of easily establishing many tumor cell lines, obtained from a number of individuals. Utilizing the methods described below, we established eight HHC cell lines from 8 patients to aid in our immunopathologic studies of human hepatoma. The methods demonstrated were designed to be simple and reproducible. They required only needle biopsy specimens less than 0.1 ml in volume. These methods should be valuable in studies of sensitivity to antitumor drugs and immunologic and biologic studies of HHC.

Materials and Methods

Biopsy Tissues

Hepatocellular carcinoma patients, with or without cirrhosis, were studied by liver scanning and peritoneoscopy. Aspirated needle liver biopsies were performed through peritoneoscopy. The largest fragments of the biopsy specimen were processed for histopathologic diagnosis (Figure 4A), and the smaller fragments were put into cold culture medium and kept at 4 C until cell culturing was done within 2 hours after the biopsy.

Medium

Ham's F-12 (GIBCO Laboratories, Grand Island, NY) with 5% lactabumin hydrolysate (GIBCO) was

Supported by the Thai National Research Council.
Accepted for publication September 6, 1984.
Address reprint requests to Kingkarn Laohathai, MD, PhD, Department of Diagnostic Immunological Research and Biochemistry, Roswell Park Memorial Institute, 666 Elm Street, Buffalo, NY 14263.

supplemented with 20% fetal calf serum (GIBCO; virus and mycoplasma tested). Antibiotics were not used.

Tissue Culturing Methods

Aspirated needle biopsy specimens often provided small fragments of tissue of a volume less than 0.1 ml. Biopsied sections that were larger than 1 mm in diameter were minced. Specimens were washed three times with culture medium before seeding (plating). In samples where the cellular inoculum was especially large, the floating cells and fragments were transferred to new cultured vessels, tubes (Leighton Culture Tube), or flasks, on successive culture days, for a period of 10 days. Transferred cells or tissue microfragments became adherent to the new cell culture vessels and grew as rapidly as the cells that were plated earlier. The medium was changed 24 hours after the initial seeding and thereafter once every 3 or 4 days. The cells were maintained in a 5% CO_2 incubator (Forma Scientific Model 3000/30004, Forma Scientific, Division of Mallinckrodt, Inc.).

Tumorigenicity Tests

Twenty-four 8-week-old SPE, nu/nu, mice were donated from breeding stock of Nihon University, Medical School, Tokyo, Japan. Sets of 3 nude mice were implanted subcutaneously with 2×10^6 cultured tumor cells from each of our eight distinct hepatocellular carcinoma lines: HHP-40, HHP-16, HHS-20, HHS-25, HHS-26, HHS-56, HHS-85, and HHS-89.

In order to observe possible phenotypic changes that might arise through the passage of these cell lines in nude mice, we utilized a biopsy of a HHS-85 human tumor xenograft from a nude mouse in the reestablishment of a permanent cell culture line (HHS-85 Nu). We utilized the same tissue culture methods as previously described.

Chromosomal Analysis

Chromosomes were prepared by the nonbanding method.[13] Twenty-four hours after adding fresh medium, confluent tumor cells in a 75-sq cm culture flask were incubated for 1 hour with 1 ml of colchicine (50 mg/l). The incubation was terminated by trypsinization for collection of a suspension of separated cells. Potassium chloride (0.075% in sterile water) was used as a hypotonic treatment. The cells were then fixed for at least 30 minutes in a mixture of methyl alcohol and glacial acetic acid (3:1). The chromosomes were analyzed after 138, 212, and 418 days of cell culturing for each of the cell lines analyzed. The mode number of chromosomes was taken from 100 cells analyzed.

Transmission Electron Microscopic Studies

The cultured cells were fixed with 2% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.4, washed three times with 0.1 M PBS at pH 7.4, and postfixed with 1% OsO_4 for 1 hour. The cells were embedded in soft agar and dehydrated stepwise with ethanol and then embedded in Epon 812. The tissues from nude mice were minced and fixed with 2% glutaraldehyde in 0.1 M PBS, pH 7.4, washed three times with 0.1 M PBS, postfixed with 1% OsO_4 for 1 hour, rinsed in Millonig's buffer, pH 7.4, at 4 C, and dehydrated stepwise with ethanol. Ultrathin sections were stained with lead acetate and uranyl acetate and observed by transmission electron microscopy.

Assay of α-Fetoprotein

Tumor cells (2×10^6) were incubated for 72 hours in the culture medium without serum. The supernatant was then concentrated 20-fold with the use of 20 M PEG (The British Drug Houses Ltd., B.D.H. Laboratory Chemical Division, London, England). The cultured media were assayed on Days 3, 20, 80, 120, 340, 420, 650, and 720 of culture. Outchterlony immunodiffusion was routinely performed for qualitative detection. Quantitative screenings were performed with the use of a commercial radioimmunoassay (α-fetoprotein Riaki II, Dinabot Radioisotope Lab. Ltd., Saitama-Ken, Japan).

Assay of Enzymes

The cell pellets were obtained from four confluent 150-sq cm flasks. After trypsinization the HHC cells were centrifuged at 700 rpm for 5 minutes and were resuspended in 1.0 ml of 0.15 M KCl, 4 C. The suspensions were homogenized with a homogenizer at 500 rpm. The possible presence of secreted enzymes was tested with the use of free culture medium after 3 days' incubation with the tumor cells. The activities of γ-glutamyl transferase (γ-GT), sorbital dehydrogenase (SDH), glutamate dehydrogenase (GlDH), aspartate transaminase (GOT), alanine transaminase (GPT), and alkaline phosphatase (Alp) were assayed kinetically by the methods of Szazs,[14] Gerlach and Hiby,[15] Schmidt,[16] Bergmeyer and Bernt,[17] and Hausamen,[18] respectively. Bilirubin was determined by the method of Jendrassik et al.[19]

Assay of Human Albumin

An ELISA was developed to quantitate albumin levels. Briefly, 100 μl of a 1:400 dilution of goat anti-human serum albumin (Miles Scientific, Naperville, Ill)

was incubated for 4 hours at room temperature in microwell plates. After washing, 50 μl of each sample (culture medium or homogenized cells) was added to the plates and incubated overnight at 4 C. The plates were washed, and horseradish peroxidase-linked anti-human albumin at 1:1000 in 0.5% bovine serum albumin (BSA) in PBS, pH 7.2, was added to the wells and incubated 1 hour at 37 C. The plates were washed, 100 μl of O-phenylenediamine solution (0.4 mg O-phenylenediamine with 10 ml of 0.01% hydrogen peroxide in 0.05 M citrate buffer, pH 5) was added, and the plates were incubated for 30 minutes at 37 C. The substrate reaction was read at 488 mm with a spectrophotometer.

Assay of HBs Antigen

Radioimmunoassay using an AUSIA II-125 Kit (Abbott Laboratories, Diagnostic Division, North Chicago, Ill) was used for the measurement of HBs antigen. Briefly, 200 μl of each sample (culture medium or homogenated cells) was incubated overnight at room temperature with plastic beads coated with guinea pig anti-HBs. After washing, 200 μl of ^{125}I-labeled HBs antigen (human) was added, and the tray was incubated in a 45 C water bath for 1 hour. The radioactivity remaining on the bead after washing was then measured with a gamma counter.

Results

Seventy biopsies were performed. From these, 28 cases were found to be adequate specimens (containing only tumor tissue), and the remaining 42 cases were inadequate due to the composition of the specimens. The inadequate specimens include those containing normal liver tissue (20 cases), necrotic tissue (13 cases), fibrotic tissue from cirrhotic portions (6 cases), or liver cells with cholestasis (3 cases). Initially, 25 of the 28 adequate specimens were maintained as early primary cultures; eventually, however, only 8 cell lines were selected to be maintained in long-term cultures.

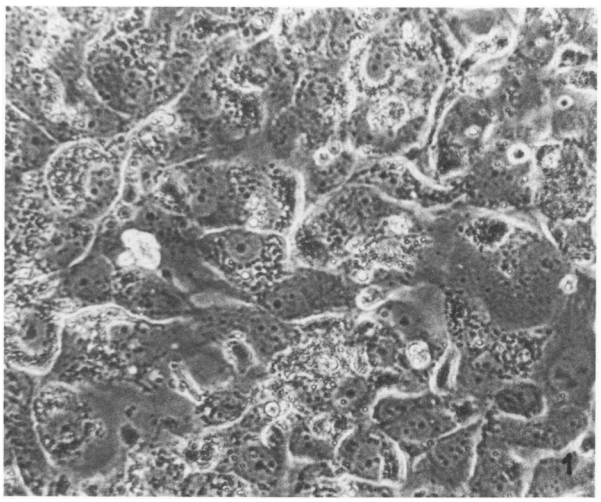
The growth rate of cultures seeded from tissues that were initially longer than 2 mm and then minced was considerably slower than smaller microfragmented specimens. Morphologically, two typical growth patterns were observed: clustered (mosaic) cells or a mixed cellular pattern, as illustrated in Figures 1, 2, and 3, respectively. Several passages after the primary culture the tumor cells grew rapidly and were able to be maintained continuously with a very low inoculum. The stationary stage was reached at about 7 to 10 days after each cell passage. The doubling time was determined to be approximately 40 hours.

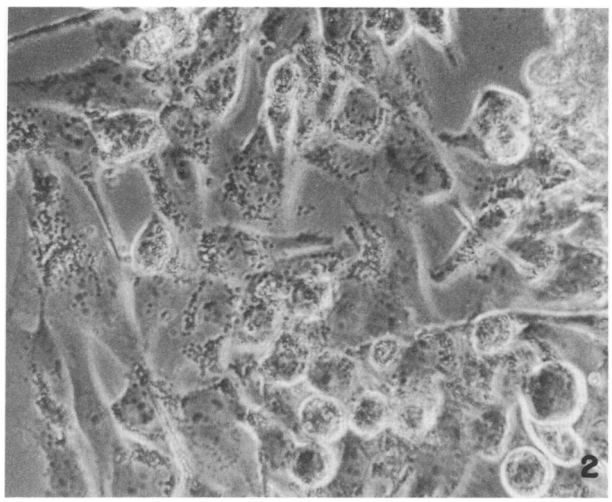
As shown in Table 1, the cells secreted human α-fetoprotein from the earliest stage of culture up until

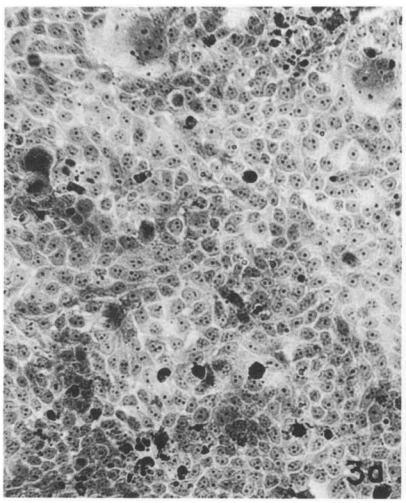
Table 1—Characteristics of Four Human Cell Lines

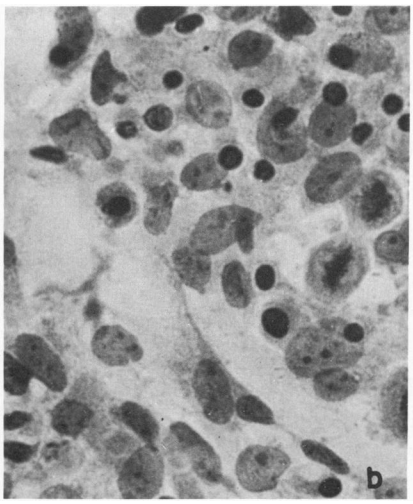
Cell lines	α-Fetoprotein			Albumin (ng/l)		HBs antigen (sera)						Ploidy designation	Chromosome mode no. (n = 100)	Abnormality
						Patient		Cell lines		Mice				
	Patient serum	Culture medium	Mouse serum	Cells*	Medium	CIE	RIA	CIE	RIA	CIE	RIA			
HHP-40	+	+	+	–	–	–	–	–	–	–	–	Hypotetraploid	76	Acrocentric Submetacentric
HHS-85	+	+	+	110	–	–	ND	–	ND	–	ND	Hyperdiploid	63	Metacentric Submetacentric Dicentric
HHS-85Nu†	ND‡			5,000		ND							ND	
HHS-89	+	+	+	ND	ND	–	ND	–	ND	–	ND	Hyperdiploid	64	Metacentric Submetacentric Dicentric
HHS-56	+	+	+	17,000	1,600	–	ND	–	ND	–	ND			ND

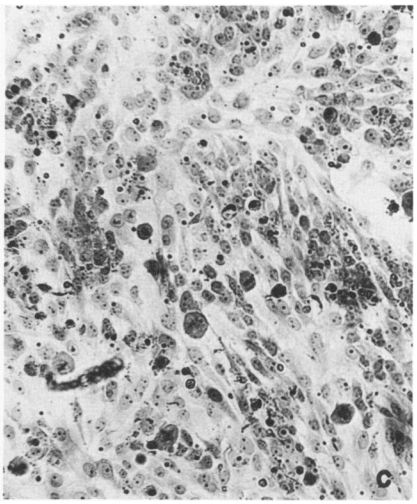
* Homogenated cells.
† HHS-85Nu, HHS-85-derived cell line after one tumor passage in nude mice.
‡ ND, not determined.

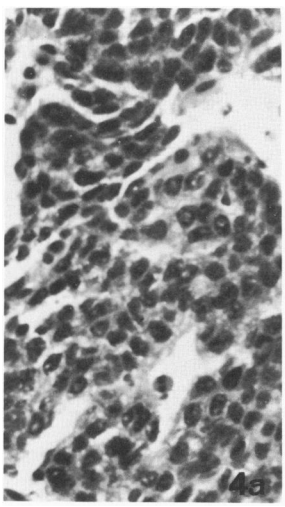


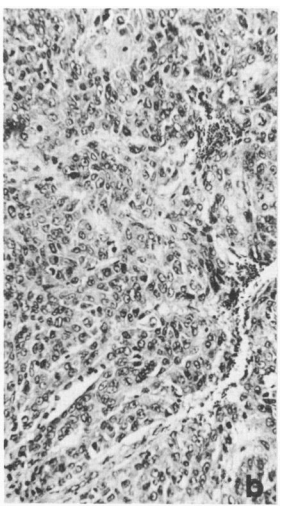












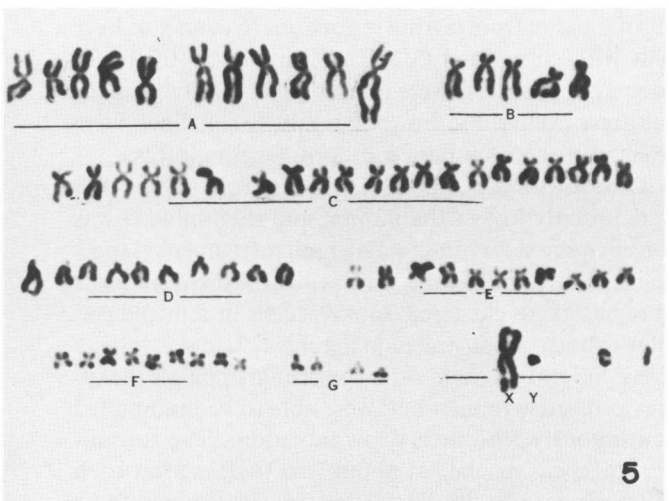


Figure 1—Cluster pattern: polygonal in shape, tight continuous growth with numerous granules in cytoplasm. (Phase contrast, × 165) **Figure 2**—Mixed pattern: polygonal and spindle in shape, loose growth. (Phase contrast, × 165) **Figure 3a**—Cluster pattern: composed of uniform cells. (H&E, × 100) **b**—Mixed pattern: hypermitotic activity. (H&E, × 200) **c**—Mixed pattern: composed of two types of cells—polygonal and spindle-shaped. (H&E, × 100) **Figure 4a**—Biopsy specimen: trabecular growth tumor. (H&E, × 200) **b**—Tumor tissue that was transplanted into nude mice. (H&E, × 100) **Figure 5**—Chromosome pattern of HHP-40 grew in a cluster pattern. (× 1000)

the time of the writing of this report (approximately 6 years). All of the cell lines were shown to be positive by the qualitative assay. In addition, up to 5.6 ng of α-fetoprotein was detected per liter of culture medium of cell lines assayed quantitatively (data not shown).

The cell lines, culture medium, and the serum of nude mice which received transplanted cell lines were all negative for the presence of the HBs antigen by counter-immunoelectropheresis assay (CIE) and by the radio-immunoassay (Table 1). However, HBs antigen was detected in the serum of 1 out of the 8 patients from which the HCC cell lines were derived.

Within 10 days after the injection, tumors were seen in 22 out of 24 nude mice. The tumors generally grew to about 3 cm in diameter, and were solid and well encapsulated. These rapidly growing tumors resulted in the death of nearly all of the mice within 2.5 months after implantation. Histologic examination showed the tumors to be carcinomas resembling the original tumors (Figure 4B).

Chromosome analysis was performed on three of the tumor lines: cell lines HHP-40, HHS-85, and HHS-89. The tumor cell lines were analyzed 138, 212, and 418 days after cultivation. HHS-89 was characterized by a hyperdiploid karyotype (64 chromosomes) including three large submetacentric and some dicentric chromosomes. In some cells a large acrocentric chromosome was observed. A similar pattern was shown in karyotype in the HHS-85 line, which was hyperdiploid (63 chromosomes), including one large metacentric, two large submetacentric, some dicentric, and one large acrocentric chromosomes in some of the cells. The HHP-40 was hypotetraploid (76 chromosomes) and exhibited a single large submetacentric chromosomal abnormality (Figure 5).

As shown in Table 2, significant amounts of normal liver cell products have been detected both in medium and cell homogenate. Gamma-GT, SDH, Alp, GLDH, GOT, GPT, and bilirubin were detected in the three cell lines assayed.

Utilizing transmission electron microscopy, the tumor cells showed prominent and irregularly shaped nuclei with very deep indentations. The rough endoplasmic reticulum was scant but associated with mitochondria. A few lysosomes and amembranous vacuoles were found in the cytoplasm. In the later passages (over 100 passages), microfilaments were found in significant numbers. All eight of the cell lines, whether growing in clusters or in mixed patterns, possessed the distinct appearance of intercellular canaliculi formation resembling bile canaliculi (Figure 6).

Discussion

Many of the specific functions of the liver, such as the production of albumin, α-fetoprotein, bilirubin, and γ-glutamyl transpeptidase, have been shown to be preserved by our hepatocellular carcinoma cell lines. Intercellular canaliculi were seen at the ultrastructural level. The cultured cells grew as solid carcinomas in nude mice. The development of these tumors in the nude mice was followed by the appearance of human α-fetoprotein in their sera. These studies indicate that the cell lines were human hepatocellular carcinoma lines and that the tumor lines maintained many of the basic functions of normal liver cells.

These experiments demonstrated that a limited amount of specimen (smaller than 0.1 ml) can be as valuable as larger tissue samples taken by opening biopsy. The successful culturing of adequate specimens was approximately 90%. These techniques could be helpful in instances where only very small amounts of tissue are available to the researcher or where only needle biopsy samples are available.

These tumor cell lines have been utilized in our development of murine monoclonal antibodies (MōAbs) that show high specificity to our HCC cell lines. These MōAbs similarly showed specific binding to several new primary HCC cell cultures. These same MōAbs demonstrated tumor specificity on human paraffin-embedded specimens using immunoperoxidate staining techniques (manuscript in preparation). We have found that because of the rapid rate at which these hepatocellular carcinoma cells generate tumors in nude mice this is

Table 2—Biochemical Characteristics of Three Cell Lines (Enzyme activities, Bilirubin and total Protein)

Medium (homogenated cells)	γ-GT (g/l)	SDH (g/l) × 10^{-5}	Alp	GlDH	GOT	GPT	Bilirubin	Protein (ng/g)
			(U/g)					
HHP-40	0.5	5.9	0.6	0	22.8	1.4	ND	6.1
	(2.2)	(42.8)	(1.4)	(0.5)	(43.2)	(0.3)	(0.16)	(5.7)
HHS-85	0	18.0	0.3	0.2	32.6	1.3	ND	4.5
	(2.0)	(17.1)	(0.7)	(0.8)	(52.0)	(2.9)	(0.45)	(4.9)
HHS-89	0.5	17.3	1.0	0.3	25.7	1.2	ND	5.3
	(1.1)	(49.6)	(1.5)	(0.8)	(67.9)	(2.0)	(0.08)	(2.5)

ND, not determined.

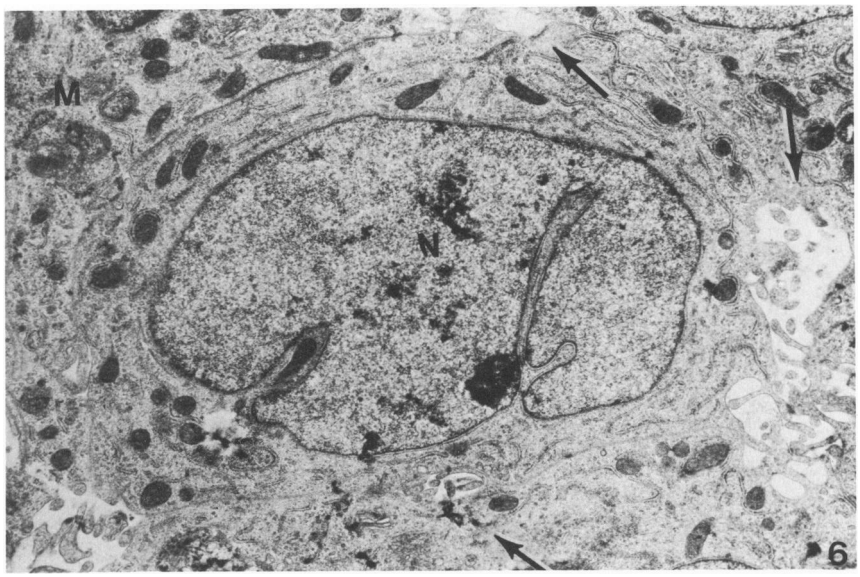


Figure 6—A cell with intercellular canaliculi (*single arrow*), surrounded by three neighboring cells. The nucleus (*N*) is large, invaginated, and has two nucleoli. Mitochondria (*M*) are scattered. (× 10,500)

an ideal model for the therapeutic testing of pharmacologic and immunologic agents (manuscript in preparation). These cell lines should also be useful for a wide variety of other studies of the nature and treatment of HHC.

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Acknowledgments

Special thanks are due to Dr. Tongtavuch Anukarahanonta, Department of Pathology, Faculty of Science, for the study of biochemical properties of our cell lines; Dr. Rudee Pleehachinda, Dr. Vipa Boonamsiri, and Ms. Chaveevan Pattanachak, of the Nuclear Medicine Section, Department of Radiology, Siriraj Hospital, for the assay of α-fetoprotein, and the staff of the Department of Gastroenterology at Siriraj Hospital, Mahidol University, for biopsy specimens.